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(54) Title: RECOMBINANT FUSION PROTEINS TO GROWTH HORMONE AND SERUM ALBUMIN

(57) Abstract

Fusion proteins of albumin and growth hormone, or fusions of variants of either, are secreted well in yeast and have increased serum and storage stability.

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RECOMBINANT FUSION PROTEINS TO GROWTH HORMONE AND SERUM ALBUMIN

Field of the invention

5   The present invention relates to recombinant fusion proteins, to growth hormone (GH), to serum albumin and to production of proteins in yeast.

Background and prior art

10   Human serum albumin (HSA), a protein of 585 amino acids, is responsible for a significant proportion of the osmotic pressure of serum and also functions as a carrier of endogenous and exogenous ligands. At present, HSA for clinical use is produced by extraction from human blood. The production of recombinant HA (rHA) in microorganisms has

15   been disclosed in EP 330 451 and EP 361 991.

The role of albumin as a carrier molecule and its inert nature are desirable properties for use as a stabiliser and transporter of polypeptides. The use of albumin as a component of a fusion protein for stabilising other proteins

20   has been disclosed in WO 93/15199, WO 93/15200, and EP 413 622. The use of N-terminal fragments of HSA for fusions to polypeptides has also been disclosed (EP 399 666). Fusion to the said polypeptide is achieved by genetic manipulation, such that the DNA coding for HSA, or a fragment thereof, is joined to the DNA coding for the said polypeptide.

25   A suitable host is then transformed or transfected with the fused nucleotide sequences, so arranged on a suitable plasmid as to express a fusion polypeptide.   Nomura *et al* (1995) attempted to express human apolipoprotein E in *S. cerevisiae* as a fusion protein with HSA or fragments of HSA, using the HSA pre-sequence to direct secretion.

30   Whilst fusion to full length HSA resulted in the secretion of low levels of

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the protein into the medium (maximum yield of 6.3 mg per litre), fusion to HSA (1-198) or HSA (1-390) did not result in secretion into the medium.

5   Human growth hormone (reviewed by Strobl and Thomas, 1994) consists of a single polypeptide of 191 amino acids, internally cross-linked by two disulphide bonds. Two molecules of hGH receptor bind each molecule of hGH to facilitate signal transduction (Cunningham et al, 1991; de Vos et al, 1992). The C-terminus of the hGH molecule is involved in binding to

10   the first receptor molecule, but the extent to which the N-terminus is involved in receptor binding is not known. The hormone is secreted from the anterior pituitary gland under hypothalamic control, and is responsible for a wide range of growth-promoting effects in the body. Clinically, hGH is used in the treatment of hypopituitary dwarfism, chronic renal

15   insufficiency in childhood, bone fractures and burns. Current methods of production of hGH for therapeutic use are by extraction from human pituitary gland, recombinant expression in Escherichia coli as disclosed in EP 127 305 (Genentech) or recombinant expression in mammalian cell culture (Zeisel et al, 1992).

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In addition, hGH has been expressed intracellularly in yeast (Tokunaga et al, 1985) and this organism may provide an alternative means of production as disclosed in EP 60 057 (Genentech). Tsiomenko et al (1994) reported the role of the yeast MFα-1 prepro leader sequence in the

25   secretion of hGH from yeast. Attachment of the pre-portion of the leader sequence to the hGH gene resulted in hGH accumulation in the periplasm and vacuoles, whilst attachment of the pro-portion to hGH resulted in expression of a non-glycosylated precursor localised inside the cell. Only when both portions of the leader sequence were attached to the hGH gene

30   was hGH secreted into the culture medium. Other secretion signals (pre-

sequences) were also ineffective unless a yeast-derived pro sequence was used, suggesting that such a pro sequence was used is critical to the efficient secretion of hGH in yeast.

5   In humans, hGH is secreted into the blood in pulses, and in the circulation has a half-life of less than 20 minutes (Haffner *et al*, 1994). Elimination of the hormone is primarily via metabolism in the liver and kidneys and is more rapid in adults than in children (Kearns *et al*, 1991). Treatment for hGH deficiency generally lasts for 6 to 24 months, during which hGH

10   is administered either three times a week intramuscularly or on a daily basis subcutaneously. Such a regimen of frequent administration is necessary because of the short half-life of the molecule.

Poznansky *et al* (1988) increased the half-life of porcine growth hormone

15   by conjugation with either porcine or human serum albumin (HSA) to form relatively large conjugates of about 180 kD. Chemical reaction using the cross-linking reagent glutaraldehyde resulted in, on average, two molecules of albumin complexed with six molecules of growth hormone. The resulting 180 kD conjugate was found to have an extended half-life

20   in the circulation of rats of 2 to 3 hours, compared to 5 minutes for unconjugated growth hormone. Activity assays showed that the conjugate retained full, and possibly increased activity *in vitro*, but was inactive *in vivo*.

25   **Summary of the invention**

The invention relates to proteins formed by the fusion of a molecule of albumin, or variants or fragments thereof, to a molecule of growth hormone or variants or fragments thereof, the fusion proteins having an

30   increased circulatory half-life over unfused growth hormone. For

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convenience, we shall refer to human albumin (HA) and human growth hormone (hGH), but the albumin and growth hormones of other vertebrates are included also. Preferably, the fusion protein comprises HA, or a variant or fragment thereof, as the N-terminal portion, with hGH

5   or a variant or fragment thereof as the C-terminal portion, so as to minimise any possible negative effects on receptor binding. Alternatively, a fusion protein comprising HA, or a variant or fragment thereof, as the C-terminal portion, with hGH or a variant or fragment thereof as the N-terminal portion, may also be capable of signal transduction. Generally,

10 the polypeptide has only one HA-derived region and one GH-derived region.

Additionally, the fusion proteins of the invention may include a linker peptide between the two fused portions to provide a greater physical

15 separation between the two moieties and thus maximise the availability of the hGH portion to bind the hGH receptor. The linker peptide may consist of amino acids such that it is flexible or more rigid.

The linker sequence may be cleavable by a protease or chemically to yield

20 the growth hormone related moiety. Preferably, the protease is one which is produced naturally by the host, for example the *S. cerevisiae* protease kex2 or equivalent proteases. Hence, a further aspect of the invention provides a process for preparing growth hormone or a variant or fragment thereof by expressing a polynucleotide which encodes a polypeptide of the

25 invention in a suitable host, cleaving the cleavable linker to yield the GH-type compound and recovering the GH-type compound from the host culture in a more pure form.

We have discovered that the polypeptides of the invention are significantly

30 more stable in solution than hGH. The latter rapidly becomes inactive

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when stored in solution at 4°C for over one month. Currently marketed hGH is sold as a freeze-dried powder.

Suitably, the fusion polypeptides are produced as recombinant molecules by secretion from yeast, a microorganism such as a bacterium, human cell line or a yeast. Preferably, the polypeptide is secreted from the host. We have found that, by fusing the hGH coding sequence to the HA coding sequence, either to the 5' end or 3' end, it is possible to secrete the fusion protein from yeast without the requirement for a yeast-derived pro sequence. This was surprising, as other workers have found that a yeast derived pro sequence was needed for efficient secretion of hGH in yeast. For example, Hiramitsu *et al* (1990, 1991) found that the N-terminal portion of the pro sequence in the *Mucor pusillus* rennin pre-pro leader was important. Other authors, using the MFα-1 signal, have always included the MFα-1 pro sequence when secreting hGH. The pro sequences were believed to assist in the folding of the hGH by acting as an intramolecular chaperone. The present invention shows that HA or fragments of HA can perform a similar function.

Hence, a particular embodiment of the invention comprises a DNA construct encoding a signal sequence effective for directing secretion in yeast, particularly a yeast-derived signal sequence (especially one which is homologous to the yeast host), and the fused molecule of the first aspect of the invention, there being no yeast-derived pro sequence between the signal and the mature polypeptide.

The *Saccharomyces cerevisiae* invertase signal is a preferred example of a yeast-derived signal.

Conjugates of the kind prepared by Poznansky *et al* (1988), in which

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separately-prepared polypeptides are joined by chemical cross-linking, are
not contemplated.

The albumin or hGH may be a variant of normal HSA/rHA (termed
5   hereinafter "HA") or hGH, respectively. By "variants" we include
insertions, deletions and substitutions, either conservative or non-
conservative, where such changes do not substantially alter one or more
of the oncotic, useful ligand-binding and non-immunogenic properties of
albumin or, in the case of hGH, its non-immunogenicity and ability to
10   bind and activate the hGH receptor. In particular, we include naturally-
occurring polymorphic variants of human albumin and fragments of human
albumin, for example those fragments disclosed in EP 322 094 (namely
HA (1-n), where n is 369 to 419). The albumin or growth hormone may
be from any vertebrate, especially any mammal, for example human, cow,
15   sheep, pig, hen or salmon. The albumin and GH parts of the fusion may
be from differing animals.

By "conservative substitutions" is intended swaps within groups such as
Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and
20   Phe, Tyr. The variant will usually have at least 75% (preferably at least
80%, 90%, 95% or 99%) sequence identity with a length of normal HA
or hGH which is the same length as the variant and which is more
identical thereto than any other length of normal HA or hGH, once the
allowance is made for deletions and insertions as is customary in this art.
25   Generally speaking, an HA variant will be at least 100 amino acids long,
preferably at least 150 amino acids long. The HA variant may consist of
or comprise at least one whole domain of HA, for example domains 1 (1-
194), 2 (195-387), 3 (388-585), 1 + 2 (1-387), 2 + 3 (195-585) or 1 +
3 (1-194, + 388-585). Each domain is itself made up of two homologous
30   subdomains namely 1-105, 120-194, 195-291, 316-387, 388-491 and 512-

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585, with flexible inter-subdomain linker regions comprising residues Lys106 to Glu199, Glu292 to Val315 and Glu492 to Ala511. Preferably, the HA part of the fusion comprises at least one subdomain or domain of HA or conservative modifications thereof. If the fusion is based on subdomains, some or all of the adjacent linker is preferably used to link to the hGH moiety. The hGH variant should have GH activity, and will generally have at least 10 amino acids, (although some authors have found activity with only 4 residues), preferably at least 20, preferably at least 50, 100, 150, 180 or 191, amino acids long, and preferably retains its cysteines for both internal disulphide bonds.

The fused molecules of the invention generally have a molecular weight of less than 100 kD, for example less than 90 kD or 70 kD. They are therefore much smaller than the 180 kD conjugates of Poznansky *et al* (referred to above), which were inactive *in vivo*. They will normally have a molecular weight of at least 20 kD, usually at least 30 kD or 50 kD. Most fall within the molecular weight range 60-90 kD.

A second main aspect of the invention provides a yeast transformed to express a fusion protein of the invention.

In addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium. Especially if the polypeptide is secreted, the medium will thus contain the polypeptide, with the cells, or without the cells if they have been filtered or centrifuged away.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*,

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Kluyveromyces lactis and *Pichia pastoris*, filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The desired protein is produced in conventional ways, for example from

5   a coding sequence inserted in the host chromosome or on a free plasmid.

The yeasts are transformed with a coding sequence for the desired protein in any of the usual ways, for example electroporation. Methods for transformation of yeast by electroporation are disclosed in Becker &

10   Guarente (1990) *Methods Enzymol.* **194**, 182.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct

15   can be grown to produce the desired polypeptide. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* **3**, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies.

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Useful yeast plasmid vectors include pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers

25   *HIS3, TRP1, LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance,

30   complementary homopolymer tracts can be added to the DNA segment to

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be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

5   Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-
10   5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large
15   molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme
20   and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International
25   Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA in accordance with the invention, if, for example, HA variants are to be prepared, is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* 239, 487-491.
30   In this method the DNA to be enzymatically amplified is flanked by two

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specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

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Exemplary genera of yeast contemplated to be useful in the practice of the present invention as hosts for expressing the fusion proteins are *Pichia (Hansenula)*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Torulaspora*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*,

10   *Debaromyces*, *Metschunikowia*, *Rhodosporidium*, *Leucosporidium*, *Botryoascus*, *Sporidiobolus*, *Endomycopsis*, and the like. Preferred genera are those selected from the group consisting of *Saccharomyces*, *Schizosaccharomyces*, *Kluyveromyces*, *Pichia* and *Torulaspora*. Examples of *Saccharomyces* spp. are *S. cerevisiae*, *S. italicus* and *S. rouxii*.

15   Examples of *Kluyveromyces* spp. are *K. fragilis*, *K. lactis* and *K. marxianus*. A suitable *Torulaspora* species is *T. delbrueckii*. Examples of *Pichia (Hansenula)* spp. are *P. angusta* (formerly *H. polymorpha*), *P. anomala* (formerly *H. anomala*) and *P. pastoris*.

20   Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference.

Suitable promoters for *S. cerevisiae* include those associated with the

25   *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α-mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GUT2* promoter, the

30   *GPD1* promoter, and hybrid promoters involving hybrids of parts of 5'

regulatory regions with parts of 5' regulatory regions of other promoters or with upstream activation sites (eg the promoter of EP-A-258 067).

Convenient regulatable promoters for use in *Schizosaccharomyces pombe*

5   are the thiamine-repressible promoter from the *nmt* gene as described by Maundrell (1990) *J. Biol. Chem.* **265**, 10857-10864 and the glucose-repressible *fbp1* gene promoter as described by Hoffman & Winston (1990) *Genetics* **124**, 807-816.

10   Methods of transforming *Pichia* for expression of foreign genes are taught in, for example, Cregg *et al* (1993), and various Phillips patents (eg US 4 857 467, incorporated herein by reference), and *Pichia* expression kits are commercially available from Invitrogen BV, Leek, Netherlands, and Invitrogen Corp., San Diego, California. Suitable promoters include

15   *AOX1* and *AOX2*.

Gleeson *et al* (1986) *J. Gen. Microbiol.* **132**, 3459-3465 include information on *Hansenula* vectors and transformation, suitable promoters being *MOX1* and *FMD1*; whilst EP 361 991, Fleer *et al* (1991) and other

20   publications from Rhône-Poulenc Rorer teach how to express foreign proteins in *Kluyveromyces* spp., a suitable promoter being *PGK1*.

The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription

25   termination and polyadenylation. Suitable 3' flanking sequences may, for example, be those of the gene naturally linked to the expression control sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae ADH1* gene is preferred.

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The desired fusion protein may be initially expressed with a secretion leader sequence, which may be any leader effective in the yeast chosen. Leaders useful in *S. cerevisiae* include that from the mating factor α polypeptide (MFα-1) and the hybrid leaders of EP-A-387 319. Such

5   leaders (or signals) are cleaved by the yeast before the mature albumin is released into the surrounding medium. Further such leaders include those of *S. cerevisiae* invertase (*SUC2*) disclosed in JP 62-096086 (granted as 91/036516), acid phosphatase (*PHO5*), the pre-sequence of MFα-1, β-glucanase (*BGL2*) and killer toxin; *S. diastaticus* glucoamylase II; *S.*

10   *carlsbergensis* α-galactosidase (*MEL1*); *K. lactis* killer toxin; and *Candida* glucoamylase.

The fusion protein of the invention or a formulation thereof may be administered by any conventional method including parenteral (eg

15   subcutaneous or intramuscular) injection or intravenous infusion. The treatment may consist of a single dose or a plurality of doses over a period of time.

Whilst it is possible for a fusion protein of the invention to be

20   administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with the fusion protein and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

25   The formulation should be non-immunogenic; vaccine-type formulations involving adjuvants are not contemplated.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of

30   pharmacy. Such methods include the step of bringing into association the

fusion protein with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary,

5   shaping the product.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic

10   with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the

15   sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders.

Preferred unit dosage formulations are those containing a daily dose or

20   unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

The fusion proteins of the invention may be used in the treatment of any condition in which growth hormone is indicated, for example isolated

25   growth hormone deficiency, panhypopituitarism, following cranial irradiation (eg in the treatment of leukaemia or brain tumours), Turner's syndrome, Down's syndrome, intrauterine growth retardation, idiopathic growth deficiency, chronic renal failure, achondroplasia, female infertility and various catabolic disorders. They may also be used in the stimulation

30   of growth, and/or enhancement of lean meat proportion, in farm animals

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such as cows, sheep, goats and pigs.

The fusion protein may be administered together with insulin-like growth factor I (IGF-I).

The dosage can be calculated on the basis of the potency of the fusion protein relative to the potency of hGH, whilst taking into account the prolonged serum half-life of the fusion proteins compared to that of native hGH.   Growth hormone is typically administered at 0.3 to 30.0 IU/kg/week, for example 0.9 to 12.0 IU/kg/week, given in three or seven divided doses for a year or more. In a fusion protein consisting of full length HA fused to full length GH, an equivalent dose in terms of units would represent a greater weight of agent but the dosage frequency can be reduced, for example to twice a week, once a week or less.

Preferred examples of the invention will now be described by way of example and with reference to the accompanying figures, in which:

Figure 1 shows the human growth hormone cDNA sequence, encoding mature hGH;

Figure 2 shows a restriction enzyme map of pHGH1;

Figure 3 shows a restriction enzyme map of pBST(+) and the DNA sequence of the polylinker;

Figure 4 shows the construction of pHGH12;

Figure 5 shows the construction of pHGH16;

Figure 6 shows the HSA cDNA sequence, more particularly the region encoding the mature protein;

Figure 7 shows the construction of pHGH14;

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Figure 8 shows the construction of pHGH38;

Figure 9 shows the construction of pHGH31;

10   Figure 10 shows the construction of pHGH58 or pHGH59 (Example 7);

Figure 11 is a scheme for constructing fusions having spacers (Example 7); and

15   Figure 12 shows the results of a pharmacokinetic study showing the clearance of ^{125}I-labelled rHA-hGH compared to that of hGH following iv injection in rats. Data are from two rats in each group, and include total radioactivity and radioactivity which could be precipitated by TCA, ie that associated with protein rather than as free ^{125}I. The calculated clearance

20   half-life for hGH was approximately 6 minutes, compared to approximately 60 minutes for the rHA-hGH fusion protein. See Example 3.

   • - hGH (total counts)

25   ■ - hGH (TCA precipitated counts)

   ▼ - rHA-hGH (total counts)

   ▲ - rHA-hGH (TCA precipitated counts).

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Detailed description of the invention

All standard recombinant DNA procedures are as described in Sambrook
et al (1989) unless otherwise stated. The DNA sequences encoding HSA

5   were derived from the cDNA disclosed in EP 201 239.

Example 1: Cloning of the hGH cDNA.

The hGH cDNA was obtained from a human pituitary gland cDNA library

10   (catalogue number HL1097v, Clontech Laboratories, Inc) by PCR
amplification. Two oligonucleotides suitable for PCR amplification of the
hGH cDNA, HGH1 and HGH2, were synthesised using an Applied
Biosystems 380B Oligonucleotide Synthesiser.

15   HGH1: 5' - CCCAAGAATTCCCTTATCCAGGC - 3'
     HGH2: 5' - GGGAAGCTTAGAAGCCACAGGATCCCTCCACAG - 3'

HGH1 and HGH2 differed from the equivalent portion of the hGH cDNA
sequence (Figure 1, Martial *et al*, 1979) by two and three nucleotides,

20   respectively, such that after PCR amplification an *EcoR*I site would be
introduced to the 5' end of the cDNA and a *Bam*HI site would be
introduced into the 3' end of the cDNA. In addition, HGH2 contained a
*Hind*III site immediately downstream of the hGH sequence.

25   PCR amplification using a Perkin-Elmer-Cetus Thermal Cycler 9600 and
a Perkin-Elmer-Cetus PCR kit, was performed using single-stranded DNA
template isolated from the phage particles of the cDNA library as follows:
10 µL phage particles were lysed by the addition of 10 µL phage lysis
buffer (280 µg/mL proteinase K in TE buffer) and incubation at 55°C for

30   15 min followed by 85°C for 15 min. After a 1 min incubation on ice,

phage debris was pelleted by centrifugation at 14,000 rpm for 3 min. The PCR mixture contained 6 μL of this DNA template, 0.1 μM of each primer and 200 μM of each deoxyribonucleotide. PCR was carried out for 30 cycles, denaturing at 94°C for 30 s, annealing at 65°C for 30 s and
5 extending at 72°C for 30 s, increasing the extension time by 1 s per cycle. Analysis of the reaction by gel electrophoresis showed a single product of the expected size (589 base pairs).

The PCR product was purified using Wizard PCR Preps DNA Purification
10 System (Promega Corp) and then digested with EcoRI and HindIII. After further purification of the EcoRI-HindIII fragment by gel electrophoresis, the product was cloned into pUC19 (GIBCO BRL) digested with EcoRI and HindIII, to give pHGH1 (Figure 2). DNA sequencing of the EcoRI-HindIII region showed that the PCR product was identical in sequence to
15 the hGH sequence (Martial et al, 1979), except at the 5' and 3' ends, where the EcoRI and BamHI sites had been introduced, respectively.

Example 2: Expression of the hGH cDNA.

20 The polylinker sequence of the phagemid pBluescribe (+) (Stratagene) was replaced by inserting an oligonucleotide linker, formed by annealing two 75-mer oligonucleotides, between the EcoRI and HindIII sites to form pBST(+) (Figure 3). The new polylinker included a unique NotI site (the full sequence in the region of the polylinker is given in Figure 3).
25

The NotI HSA expression cassette of pAYE309 (EP 431 880) comprising the PRB1 promoter, DNA encoding the HSA/MFα-1 hybrid leader sequence, DNA encoding HSA and the ADH1 terminator, was transferred to pBST(+) to form pHA1 (Figure 4). The HSA coding sequence was
30 removed from this plasmid by digestion with HindIII followed by

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religation to form pHA2 (Figure 4).

Cloning of the hGH cDNA, as described in Example 1, provided the hGH
coding region lacking the pro-hGH sequence and the first 8 base pairs (bp)
5   of the mature hGH sequence. In order to construct an expression plasmid
for secretion of hGH from yeast, a yeast promoter, signal peptide and the
first 8 bp of the hGH sequence were attached to the 5' end of the cloned
hGH sequence as follows:

10   The HindIII-SfaNI fragment from pHA1 was attached to the 5' end of the
EcoRI-HindIII fragment from pHGH1 via two synthetic oligonucleotides,
HGH3 and HGH4:

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     HGH3:  5' - GATAAAGATTCCCAAC - 3'
15   HGH4:  5' - AATTGTTGGGAATCTTT - 3'
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The HindIII fragment so formed was cloned into HindIII-digested pHA2
to make pHGH2 (Figure 4), such that the hGH cDNA was positioned
downstream of the PRB1 promoter and HSA/MFα-1 fusion leader
20   sequence (WO 90/01063). The NotI expression cassette contained in
pHGH2, which included the ADH1·terminator downstream of the hGH
cDNA, was cloned into NotI-digested pSAC35 (Sleep et al, 1990) to make
pHGH12 (Figure 4). This plasmid comprised the entire 2μm plasmid to
provide replication functions and the LEU2 gene for selection of
25   transformants.

pHGH12 was introduced into S. cerevisiae DB1 (Sleep et al, 1990) by
transformation and individual transformants were grown for 3 days at
30°C in 10 mL YEPD (1% $^{w}/_{v}$ yeast extract, 2% $^{w}/_{v}$ peptone, 2% $^{w}/_{v}$
30   dextrose). After centrifugation of the cells, the supernatants were

examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were found to contain protein which was of the expected size and which was recognised by anti-hGH antiserum (Sigma, Poole, UK) on Western blots.

5

Example 3: Cloning and expression of an HSA-hGH fusion protein.

In order to fuse the HSA cDNA to the 5' end of the hGH cDNA, the pHA1 *Hind*III-*Bsu*36I fragment (containing most of the HSA cDNA) was

10 joined to the pHGH1 *Eco*RI-*Hind*III fragment (containing most of the hGH cDNA) via two oligonucleotides, HGH7 and HGH8:


```
HGH7:   5' - TTAGGCTTATTCCCAAC - 3'
HGH8:   5' - AATTGTTGGGAATAAGCC - 3'
```

15

The *Hind*III fragment so formed was cloned into pHA2 digested with *Hind*III to make pHGH10 (Figure 5), and the *Not*I expression cassette of this plasmid was cloned into *Not*I-digested pSAC35 to make pHGH16 (Figure 5).

20

pHGH16 was used to transform *S. cerevisiae* DB1 and supernatants of cultures were analysed as in Example 2. A predominant band was observed that had a molecular weight of approximately 88 kD, corresponding to the combined masses of HA and hGH. Western blotting

25 using anti-HSA and anti-hGH antisera (Sigma) confirmed the presence of the two constituent parts of the fusion protein.

The fusion protein was purified from culture supernatant by cation exchange chromatography, followed by anion exchange and gel

30 permeation chromatography. Analysis of the N-terminus of the protein by

20

amino acid sequencing confirmed the presence of the expected albumin
sequence.

An *in vitro* growth hormone activity assay (Ealey *et al*, 1995) indicated

5   that the fusion protein possessed full hGH activity, but that the potency
was reduced compared to the hGH standard. In a hypophysectomised rat
weight gain model, performed essentially as described in the European
Pharmacopoeia (1987, monograph 556), the fusion molecule was more
potent than hGH when the same number of units of activity (based on the

10   above *in vitro* assay) were administered daily. Further experiments in
which the fusion protein was administered once every four days showed
a similar overall growth response to a daily administration of hGH.
Pharmacokinetic experiments in which ^{125}I-labelled protein was
administered to rats indicated an approximately ten-fold increase in

15   circulatory half life for the fusion protein compared to hGH (Fig 12).

A similar plasmid was constructed in which DNA encoding the
S.cerevisiae invertase *(SUC2)* leader sequence replaced the sequence for
the hybrid leader, such that the encoded leader and the junction with the

20   HSA sequence were as follows:

MLLQAFLFLLAGFAAKISA ↓ DAHKS.....
            Invertase leader   HSA

25   On introduction into *S.cerevisiae* DB1, this plasmid directed the
expression and secretion of the fusion protein at a level similar to that
obtained with pHGH16. Analysis of the N-terminus of the fusion protein
indicated precise and efficient cleavage of the leader sequence from the
mature protein.

30

21

Example 4:   Cloning and expression of an hGH-HSA fusion protein.

In order to fuse the hGH cDNA to the 5′ end of the HSA cDNA (Figure

6), the HSA cDNA was first altered by site-directed mutagenesis to

5   introduce an *Eco*NI site near the 5′ end of the coding region. This was

done by the method of Kunkel *et al* (1987) using single-stranded DNA

template prepared from pHA1 and a synthetic oligonucleotide, LEU4:


```
LEU4:   5' - GAGATGCACACCTGAGTGAGG - 3'
```
10

Site-directed mutagenesis using this oligonucleotide changed the coding

sequence of the HSA cDNA from Lys4 to Leu4 (K4L). However, this

change was repaired when the hGH cDNA was subsequently joined at the

5′ end by linking the pHGH2 *Not*I-*Bam*HI fragment to the *Eco*NI-*Not*I

15   fragment of the mutated pHA1, via the two oligonucleotides HGH5 and

HGH6:


```
HGH5:   5' - GATCCTGTGGCTTCGATGCACACAAGA - 3'
HGH6:   5' - CTCTTGTGTGCATCGAAGCCACAG - 3'
```
20

The *Not*I fragment so formed was cloned into *Not*I-digested pSAC35 to

make pHGH14 (Figure 7). pHGH14 was used to transform *S. cerevisiae*

DB1 and supernatants of cultures were analysed as in Example 2. A

predominant band was observed that had a molecular weight of

25   approximately 88 kD, corresponding to the combined masses of hGH and

HA. Western blotting using anti-HSA and anti-hGH antisera confirmed

the presence of the two constituent parts of the fusion protein.

The fusion protein was purified from culture supernatant by cation

30   exchange chromatography, followed by anion exchange and gel

permeation chromatography. Analysis of the N-terminus of the protein by

22

amino acid sequencing confirmed the presence of the expected hGH
sequence.

In vitro studies showed that the fusion protein retained hGH activity, but

5   was significantly less potent than a fusion protein comprising full-length
HA (1-585) as the N-terminal portion and hGH as the C-terminal portion,
as described in Example 3.

Example 5:   Construction of plasmids for the expression of hGH

10   **fusions to domains of HSA.**

Fusion polypeptides were made in which the hGH molecule was fused to
the first two domains of HA (residues 1 to 387).   Fusion to the N-
terminus of hGH was achieved by joining the pHA1 *Hind*III-*Sap*I

15   fragment, which contained most of the coding sequence for domains 1 and
2 of HA, to the pHGH1 *Eco*RI-*Hind*III fragment, via the oligonucleotides
HGH11 and HGH12:

```
     HGH11:      5' - TGTGGAAGAGCCTCAGAATTTATTCCCAAC - 3'
20   HGH12:      5' - AATTGTTGGGAATAAATTCTGAGGCTCTTCC - 3'
```

The *Hind*III fragment so formed was cloned into *Hind*III-digested pHA2
to make pHGH37 (Fig 8) and the *Not*I expression cassette of this plasmid
was cloned into *Not*I-digested pSAC35.   The resulting plasmid, pHGH38

25   (Fig 8), contained an expression cassette that was found to direct secretion
of the fusion polypeptide into the supernatant when transformed into *S.
cerevisiae* DB1.   Western blotting using anti-HSA and anti-hGH antisera
confirmed the presence of the two constituent parts of the fusion protein.

30   The fusion protein was purified from culture supernatant by cation

23

exchange chromatography followed by gel permeation chromatography.
In vivo studies with purified protein indicated that the circulatory half-life
was longer than that of hGH, and similar to that of a fusion protein
comprising full-length HA (1-585) as the N-terminal portion and hGH as

5   the C-terminal portion, as described in Example 3. *In vitro* studies
showed that the fusion protein retained hGH activity.

Using a similar strategy as detailed above, a fusion protein comprising the
first domain of HA (residues 1-194) as the N-terminal portion and hGH

10   as the C-terminal portion, was cloned and expressed in *S. cerevisiae* DB1.
Western blotting of culture supernatant using anti-HSA and anti-hGH
antisera confirmed the presence of the two constituent parts of the fusion
protein.

15   **Example 6: Expression of hGH by introducing a cleavage site between
HSA and hGH.**

Introduction of a peptide sequence that is recognised by the Kex2 protease,
between the HA-hGH fusion protein, allows secretion of hGH.   A

20   sequence encoding Ser Leu Asp Lys Arg was introduced using two
oligonucleotides, HGH14 and HGH15:


```
HGH14:     5' - TTAGGCTTAAGCTTGGATAAAAGATTCCCAAC - 3'
HGH15:     5' - AATTGTTGGGAATCTTTTATCCAAGCTTAAGCC - 3'
```
25
These were used to join the pHA1 *Hind*III-*Bsu*36I fragment to the pHGH1
*Eco*RI-*Hind*III fragment, which were then cloned into *Hind*III-digested
pHA2 to make pHGH25 (Fig 9). The *Not*I expression cassette of this
plasmid was cloned into *Not*I-digested pSAC35 to make pHGH31 (Figure

30   9).

24

S. cerevisiae DB1 transformed with pHGH31 was found to secrete two major species, as determined by SDS-PAGE analysis of culture supernatants. The two species had molecular weights of approximately 66 kD, corresponding to (full length) HA, and 22 kD, corresponding to (full

5   length) hGH, indicating *in vivo* cleavage of the fusion protein by the Kex2 protease, or an equivalent activity. Western blotting using anti-HSA and anti-hGH antisera confirmed the presence of the two separate species. N-terminal sequence analysis of the hGH moiety confirmed the precise and efficient cleavage from the HA moiety.

10

The hGH moiety was purified from culture supernatant by anion exchange chromatography followed by gel permeation chromatography. *In vitro* studies with the purified hGH showed that the protein was active and fully potent.

15

Using a similar strategy, fusion proteins comprising either the first domain of HA (residues 1-194) or the first two domains of HA (residues 1-387), followed by a sequence recognised by the Kex2p protease, followed by the hGH cDNA, were cloned and expressed in *S. cerevisiae* DB1. Western

20   blotting of culture supernatant using anti-HSA and anti-hGH antisera confirmed the presence of the two separate species.

Example 7: Fusion of HSA to hGH using a flexible linker sequence

25   Flexible linkers, comprising repeating units of [Gly-Gly-Gly-Gly-Ser]$_n$, where n was either 2 or 3, were introduced between the HSA and hGH fusion protein by cloning of the oligonucleotides HGH16, HGH17, HGH18 and HGH19:

25

HGH16:5'-TTAGGCTTAGGTGGCGGTGGATCCGGCGGTGGTGGATCTTTCCCA
AC-3'

HGH17:5'-AATTGTTGGGAAAGATCCACCACCGCCGGATCCACCGCCACCTAA
GCC-3'

5   HGH18:5'-TTAGGCTTAGGCGGTGGTGGATCTGGTGGCGGCGGATCTGGTGGC
GGTGGATCCTTCCCAAC-3'

HGH19:   5'-AATTGTTGGGAAGGATCCACCGCCACCAGATCCGCCGCCACCA
GATCCACCACCGCCTAAGCC-3'

10   Annealing of HGH16 with HGH17 resulted in n=2, while HGH18
annealed to HGH19 resulted in n=3. After annealing, the double-stranded
oligonucleotides were cloned with the EcoRI-Bsu36I fragment isolated
from pHGH1 into Bsu36I-digested pHGH10 to make pHGH56 (where
n=2) and pHGH57 (where n=3) (Figure 10). The NotI expression
15   cassettes from these plasmids were cloned into NotI-digested pSAC35 to
make pHGH58 and pHGH59, respectively.

Cloning of the oligonucleotides to make pHGH56 and pHGH57 introduced
a BamHI site in the linker sequences, as shown in Figure 11. It was
20   therefore possible to construct linker sequences in which n=1 and n=4,
by joining either the HindIII-BamHI fragment from pHGH56 to the
BamHI-HindIII fragment from pHGH57 (making n=1), or the HindIII-
BamHI fragment from pHGH57 to the BamHI-HindIII fragment from
pHGH56 (making n=2). Cloning of these fragments into the HindIII site
25   of pHA2 (described in Example 2), resulted in pHGH60 (n=1) and
pHGH61 (n=4) (see Figure 11). The NotI expression cassettes from
pHGH60 and pHGH61 were cloned into NotI-digested pSAC35 to make
pHGH62 and pHGH63, respectively.

30   Transformation of S.cerevisiae with pHGH58, pHGH59, pHGH62 and
pHGH63 resulted in transformants that secreted the fusion polypeptides

26

into the supernatant.

Western blotting using anti-HSA and anti-hGH antisera confirmed the
presence of the two constituent parts of the fusion proteins.

5

The fusion proteins were purified from culture supernatant by cation
exchange chromatography, followed by anion exchange and gel
permeation chromatography. Analysis of the N-termini of the proteins by
amino acid sequencing confirmed the presence of the expected albumin

10   sequence. Analysis of the purified proteins by electrospray mass
spectrometry confirmed an increase in mass of 315 D (n=1), 630 D
(n=2), 945 D (n=3) and 1260 D (n=4) compared to the HSA-hGH
fusion protein described in Example 3, as expected. The purified protein
was found to be active *in vitro*.

27

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CLAIMS

1.   A polypeptide consisting of a continuous region of amino acids joined together by peptide bonds and comprising a first region of at least 10 amino acids which has at least 75% sequence identity with a same-length region of growth hormone (GH) and a second region of at least 10 amino acids which has at least 75% sequence identity with a same-length region of serum albumin.

2.   A polypeptide according to Claim 1 in which each of said first and second regions has at least 95% sequence identity with the said lengths of GH and albumin, respectively.

3.   A polypeptide according to Claim 2 wherein each of said first and second regions is at least 50 amino acids long.

4.   A polypeptide according to any one of the preceding claims wherein the first region consists of uninterrupted amino acids of the C-terminus of GH, or a conservative modification thereof, and the polypeptide binds and activates the GH receptor.

5.   A polypeptide according to Claim 4 wherein the first region is 191 amino acids long.

6.   A polypeptide according to any one of the preceding claims wherein the albumin and/or the growth hormone is human.

7.   A polypeptide according to any one of the preceding claims wherein the second region comprises at least one uninterrupted domain of albumin or a conservative modification thereof.

8.   A polypeptide according to Claim 7 wherein the second region comprises uninterrupted amino acids 1-105, 120-194, 195-291, 316-387, 388-491, 512-585, 1-194, 195-387, 388-585, 1-387 or 195-585 of human albumin or a conservative modification thereof.

5

9.   A polypeptide according to any one of the preceding claims wherein the N-terminus of the polypeptide comprises the said first region and the C-terminus comprises the said second region.

10   10.   A polypeptide according to any one of Claims 1 to 8 wherein the N-terminus of the polypeptide comprises the said second region and the C-terminus comprises the said first region.

11.   A polypeptide according to any one of the preceding claims which

15   consists of the said first and second regions, optionally with further amino acids or other compounds added to either end of the polypeptide.

12.   A microbial culture medium comprising transformed cells and a polypeptide according to any one of the preceding claims.

20

13.   A microbial culture medium comprising a polypeptide according to any one of Claims 1 to 11.

14.   A polynucleotide encoding a polypeptide according to any one of

25   Claims 1 to 11.

15.   A microbial host comprising a polynucleotide according to Claim 14 arranged for expression in the host.

30   16.   A host according to Claim 15 wherein the polypeptide is secreted

30

from the host.

17. A process for obtaining a polypeptide according to any one of Claims 1 to 11 comprising culturing a host according to Claim 15 or 16 and purifying the polypeptide.

18. A pharmaceutical formulation comprising a polypeptide according to any one of Claims 1 to 11 and a pharmaceutically acceptable carrier.

19. A method of treating a patient having a condition which is treatable with growth hormone, the method comprising administering to the patient a said-condition-alleviating, non-toxic amount of a polypeptide according to any one of Claims 1 to 11.

20. A method of increasing beyond normal the growth rate of, or ratio of lean meat to fat meat in, an animal comprising administering to the animal an effective amount of a polypeptide according to any one of Claims 1 to 11.

21. A process for producing growth hormone, comprising expressing a polynucleotide according to Claim 14 in a microbial host, preferably yeast, the polypeptide encoded thereby having a site between the said first and second regions which site is cleavable by an enzyme present in the host, or by any other enzyme, or chemically.

```
 F   P   T   I   P   L   S   R   L   F   D   N   A   M   L   R   A   H   R
TTC CCA ACC ATT CCC TTA TCC AGG CTT TTT GAC AAC GCT ATG CTC CGC GCC CAT CGT
                                                                ^50

 L   H   Q   L   A   F   D   T   Y   Q   E   F   E   E   A   Y   I   P   K
CTG CAC CAG CTG GCC TTT GAC ACC TAC CAG GAG TTT GAA GAA GCC TAT ATC CCA AAG
                                                        ^100

 E   Q   K   Y   S   F   L   Q   N   P   Q   T   S   L   C   F   S   E   S
GAA CAG AAG TAT TCA TTC CTG CAG AAC CCC CAG ACC TCC CTC TGT TTC TCA GAG TCT
                                            ^150

 I   P   T   P   S   N   R   E   E   T   Q   Q   K   S   N   L   E   L   L
ATT CCG ACA CCC TCC AAC AGG GAG GAA ACA CAA CAG AAA TCC AAC CTA GAG CTG CTC
                                        ^200

 R   I   S   L   L   L   I   Q   S   W   L   E   P   V   Q   S   L   R   S
CGC ATC TCC CTG CTG CTC ATC CAG TCG TGG CTG GAG CCC GTG CAG TCC CTC AGG AGT
                            ^250

 V   F   A   N   S   L   V   Y   G   A   S   D   S   N   V   Y   D   L   L
GTC TTC GCC AAC AGC CTG GTG TAC GGC GCC TCT GAC AGC AAC GTC TAT GAC CTC CTA
                    ^300

 K   D   L   E   E   G   I   Q   T   L   M   G   R   L   E   D   G   S   P
AAG GAC CTA GAG GAA GGC ATC CAA ACG CTG ATG GGG AGG CTG GAA GAT GGC AGC CCC
            ^350

 R   T   G   Q   I   F   K   Q   T   Y   S   K   F   D   T   N   S   H   N
CGG ACT GGG CAG ATC TTC AAG CAG ACC TAC AGC AAG TTC GAC ACA AAC TCA CAC AAC
                                                                ^450
^400
 D   D   A   L   L   K   N   Y   G   L   L   Y   C   F   R   K   D   M   D
GAT GAC GCA CTA CTC AAG AAC TAC GGG CTG CTC TAC TGC TTC AGG AAG GAC ATG GAC
                                                        ^500

 K   V   E   T   F   L   R   I   V   Q   C   R   S   V   E   G   S   C   G
AAG GTC GAG ACA TTC CTG CGC ATC GTG CAG TGC CGC TCT GTG GAG GGC AGC TGT GGC
                                            ^550

 F   .
TTC TAG
```

Figure 1. Human growth hormone cDNA sequence, encoding
         mature hGH.

Figure 2

                    AvrII   SfiI   NotI   SalI
        ... AAG CTC CTA GGC CCG GGC GGC CGC AAG CTT GTC GAC GCT AGC
                              SmaI   HindIII   NheI

            PstI   BglII   NarI
        TGC AGA AGG ATC CAG ATC TCG AGG CGC CAT CGA TAA TTC ...
                    BamHI   XhoI   ClaI

Figure 3

Figure 4

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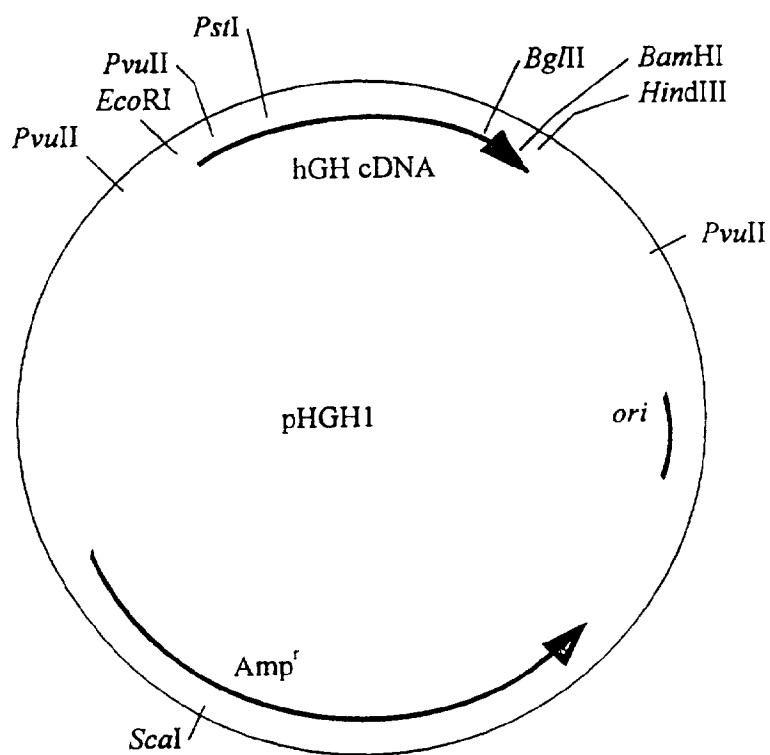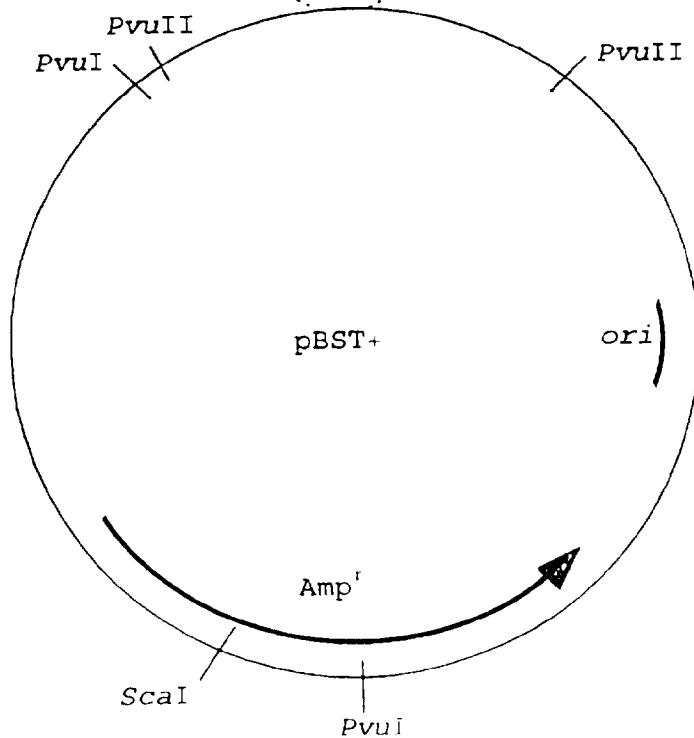
Figure 5

```
      D   A   H   K   S   E   V   A   H   R   F   K   D   L   G   E   E   N   F
      GAT GCA CAC AAG AGT GAG GTT GCT CAT CGG TTT AAA GAT TTG GGA GAA GAA AAT TTC
      K   A   L   V   L   I   A   F   A   Q   Y   L   Q   Q   C   P   F   E   D
      AAA GCC TTG GTG TTG ATT GCC TTT GCT CAG TAT CTT CAG CAG TGT CCA TTT GAA GAT
                                                                      ^100

      H   V   K   L   V   N   E   V   T   E   F   A   K   T   C   V   A   D   E
      CAT GTA AAA TTA GTG AAT GAA GTA ACT GAA TTT GCA AAA ACA TGT GTT GCT GAT GAG
      S   A   E   N   C   D   K   S   L   H   T   L   F   G   D   K   L   C   T
      TCA GCT GAA AAT TGT GAC AAA TCA CTT CAT ACC CTT TTT GGA GAC AAA TTA TGC ACA
                                          ^200

      V   A   T   L   R   E   T   Y   G   E   M   A   D   C   C   A   K   Q   E
      GTT GCA ACT CTT CGT GAA ACC TAT GGT GAA ATG GCT GAC TGC TGT GCA AAA CAA GAA
      P   E   R   N   E   C   F   L   Q   H   K   D   D   N   P   N   L   P   R
      CCT GAG AGA AAT GAA TGC TTC TTG CAA CAC AAA GAT GAC AAC CCA AAC CTC CCC CGA
                      ^300

      L   V   R   P   E   V   D   V   M   C   T   A   F   H   D   N   E   E   T
      TTG GTG AGA CCA GAG GTT GAT GTG ATG TGC ACT GCT TTT CAT GAC AAT GAA GAG ACA
      F   L   K   K   Y   L   Y   E   I   A   R   R   H   P   Y   F   Y   A   P
      TTT TTG AAA AAA TAC TTA TAT GAA ATT GCC AGA AGA CAT CCT TAC TTT TAT GCC CCG
      ^400
      E   L   L   F   F   A   K   R   Y   K   A   A   F   T   E   C   C   Q   A
      GAA CTC CTT TTC TTT GCT AAA AGG TAT AAA GCT GCT TTT ACA GAA TGT TGC CAA GCT
                                                                  ^500

      A   D   K   A   A   C   L   L   P   K   L   D   E   L   R   D   E   G   K
      GCT GAT AAA GCT GCC TGC CTG TTG CCA AAG CTC GAT GAA CTT CGG GAT GAA GGG AAG
      A   S   S   A   K   Q   R   L   K   C   A   S   L   Q   K   F   G   E   R
      GCT TCG TCT GCC AAA CAG AGA CTC AAG TGT GCC AGT CTC CAA AAA TTT GGA GAA AGA
                                      ^600

      A   F   K   A   W   A   V   A   R   L   S   Q   R   F   P   K   A   E   F
      GCT TTC AAA GCA TGG GCA GTA GCT CGC CTG AGC CAG AGA TTT CCC AAA GCT GAG TTT
      A   E   V   S   K   L   V   T   D   L   T   K   V   H   T   E   C   C   H
      GCA GAA GTT TCC AAG TTA GTG ACA GAT CTT ACC AAA GTC CAC ACG GAA TGC TGC CAT
                      ^700

      G   D   L   L   E   C   A   D   D   R   A   D   L   A   K   Y   I   C   E
      GGA GAT CTG CTT GAA TGT GCT GAT GAC AGG GCG GAC CTT GCC AAG TAT ATC TGT GAA
      N   Q   D   S   I   S   S   K   L   K   E   C   C   E   K   P   L   L   E
      AAT CAA GAT TCG ATC TCC AGT AAA CTG AAG GAA TGC TGT GAA AAA CCT CTG TTG GAA
      ^800
      K   S   H   C   I   A   E   V   E   N   D   E   M   P   A   D   L   P   S
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                                              ^900
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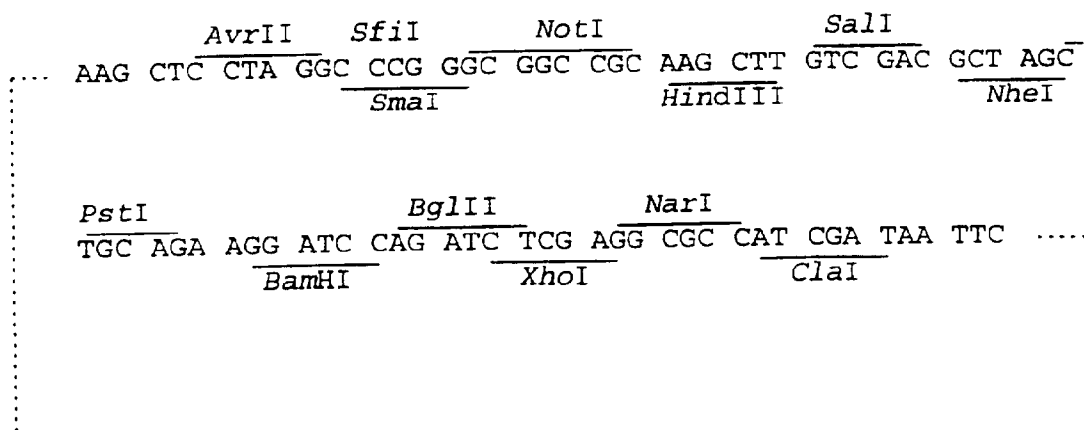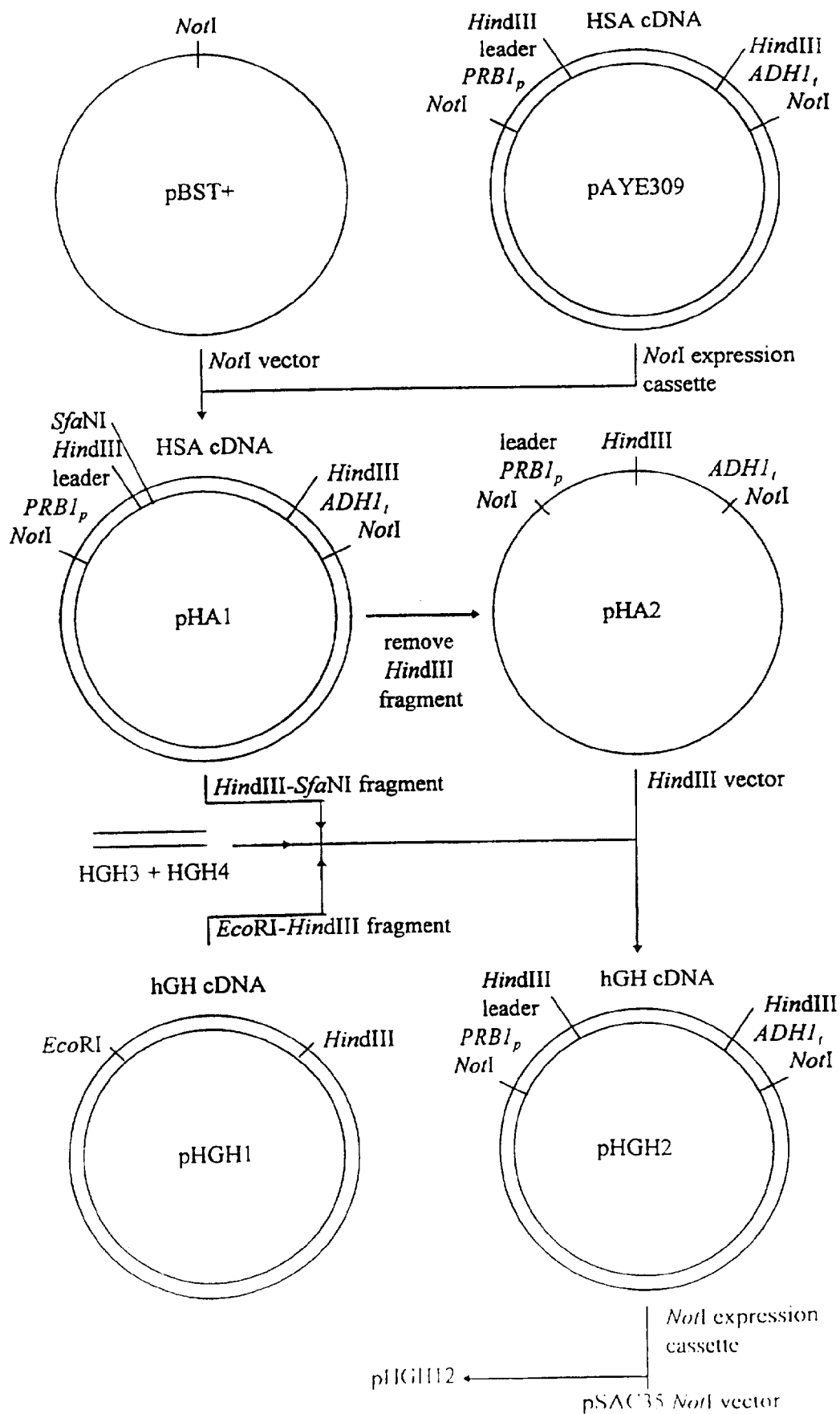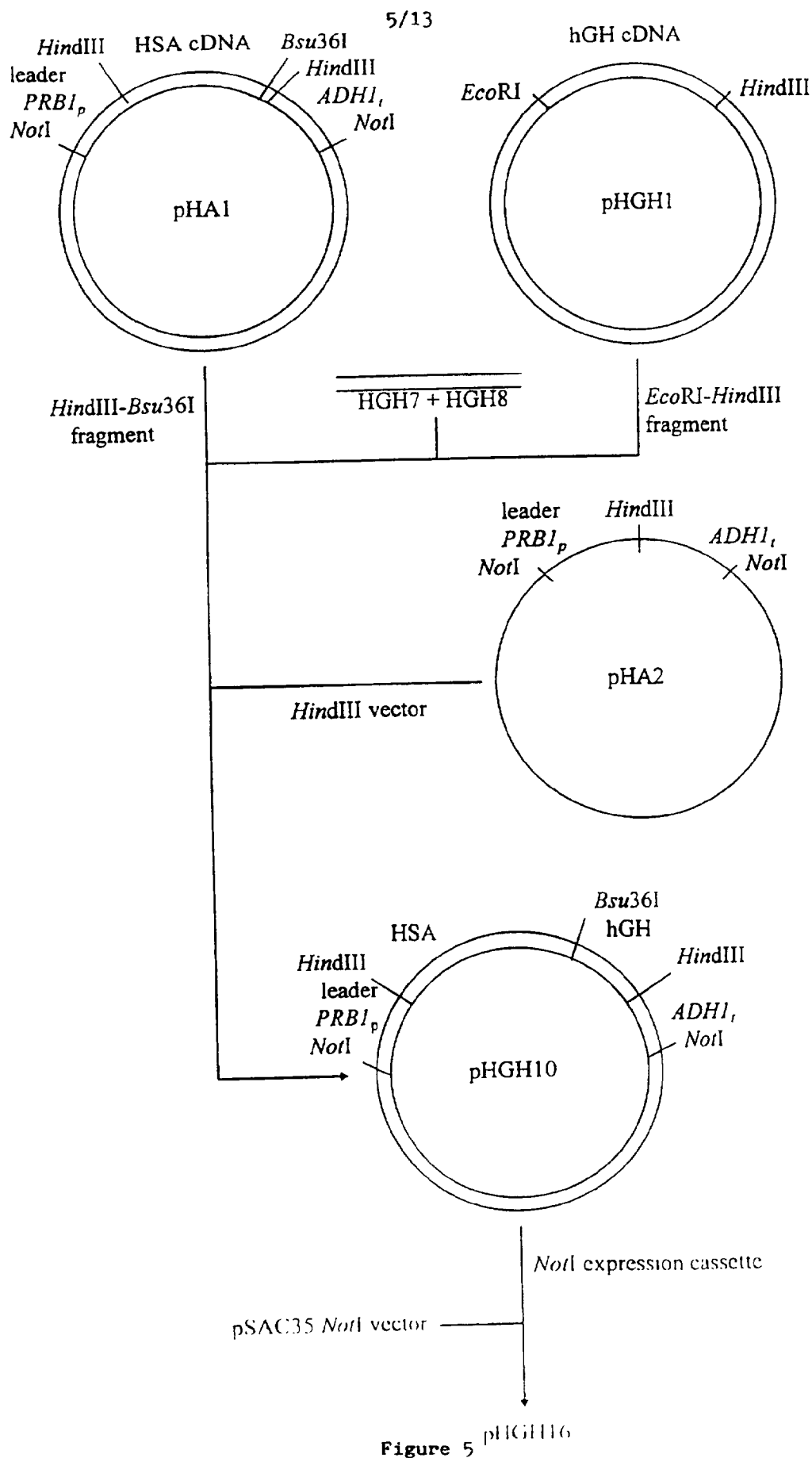
Figure 6. HSA cDNA sequence, showing the region encoding
the mature protein.

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```
L   A   A   D   F   V   E   S   K   D   V   C   K   N   Y   A   E   A   K
TTA GCT GCT GAT TTT GTT GAA AGT AAG GAT GTT TGC AAA AAC TAT GCT GAG GCA AAG
D   V   F   L   G   M   F   L   Y   E   Y   A   R   R   H   P   D   Y   S
GAT GTC TTC CTG GGC ATG TTT TTG TAT GAA TAT GCA AGA AGG CAT CCT GAT TAC TCT
                                            ^1000
V   V   L   L   L   R   L   A   K   T   Y   E   T   T   L   E   K   C   C
GTC GTG CTG CTG CTG AGA CTT GCC AAG ACA TAT GAA ACC ACT CTA GAG AAG TGC TGT
A   A   A   D   P   H   E   C   Y   A   K   V   F   D   E   F   K   P   L
GCC GCT GCA GAT CCT CAT GAA TGC TAT GCC AAA GTG TTC GAT GAA TTT AAA CCT CTT
                        ^1100
V   E   E   P   Q   N   L   I   K   Q   N   C   E   L   F   E   Q   L   G
GTG GAA GAG CCT CAG AAT TTA ATC AAA CAA AAT TGT GAG CTT TTT GAG CAG CTT GGA
E   Y   K   F   Q   N   A   L   L   V   R   Y   T   K   K   V   P   Q   V
GAG TAC AAA TTC CAG AAT GCG CTA TTA GTT CGT TAC ACC AAG AAA GTA CCC CAA GTG
 ^1200
S   T   P   T   L   V   E   V   S   R   N   L   G   K   V   G   S   K   C
TCA ACT CCA ACT CTT GTA GAG GTC TCA AGA AAC CTA GGA AAA GTG GGC AGC AAA TGT
                                                            ^1300
C   K   H   P   E   A   K   R   M   P   C   A   E   D   Y   L   S   V   V
TGT AAA CAT CCT GAA GCA AAA AGA ATG CCC TGT GCA GAA GAC TAT CTA TCC GTG GTC
L   N   Q   L   C   V   L   H   E   K   T   P   V   S   D   R   V   T   K
CTG AAC CAG TTA TGT GTG TTG CAT GAG AAA ACG CCA GTA AGT GAC AGA GTC ACC AAA
                                        ^1400
C   C   T   E   S   L   V   N   R   R   P   C   F   S   A   L   E   V   D
TGC TGC ACA GAA TCC TTG GTG AAC AGG CGA CCA TGC TTT TCA GCT CTG GAA GTC GAT
E   T   Y   V   P   K   E   F   N   A   E   T   F   T   F   H   A   D   I
GAA ACA TAC GTT CCC AAA GAG TTT AAT GCT GAA ACA TTC ACC TTC CAT GCA GAT ATA
                ^1500
C   T   L   S   E   K   E   R   Q   I   K   K   Q   T   A   L   V   E   L
TGC ACA CTT TCT GAG AAG GAG AGA CAA ATC AAG AAA CAA ACT GCA CTT GTT GAG CTC
V   K   H   K   P   K   A   T   K   E   Q   L   K   A   V   M   D   D   F
GTG AAA CAC AAG CCC AAG GCA ACA AAA GAG CAA CTG AAA GCT GTT ATG GAT GAT TTC
 ^1600
A   A   F   V   E   K   C   C   K   A   D   D   K   E   T   C   F   A   E
GCA GCT TTT GTA GAG AAG TGC TGC AAG GCT GAC GAT AAG GAG ACC TGC TTT GCC GAG
                                                            ^1700
E   G   K   K   L   V   A   A   S   Q   A   A   L   G   L
GAG GGT AAA AAA CTT GTT GCT GCA AGT CAA GCT GCC TTA GGC TTA TAA
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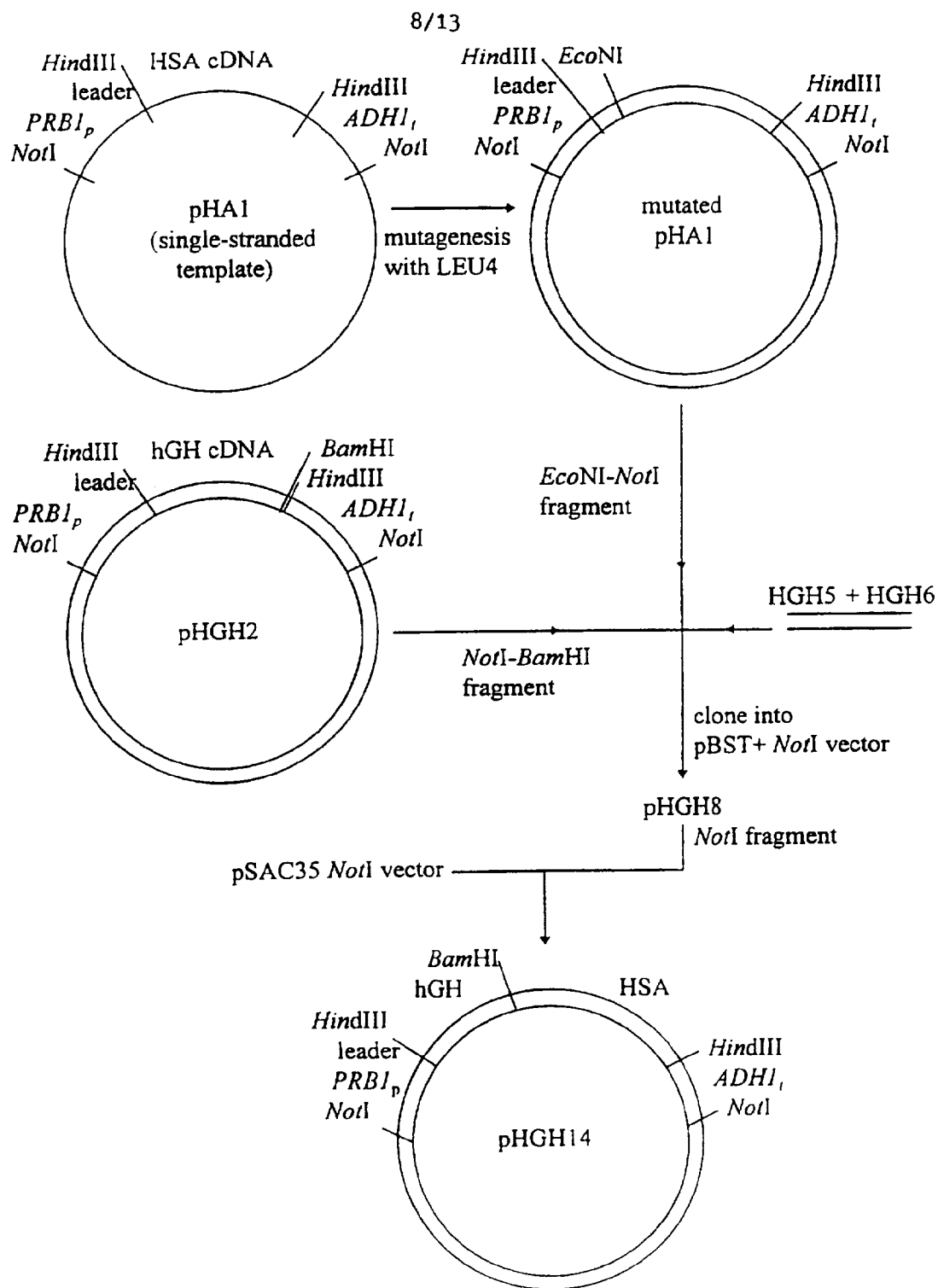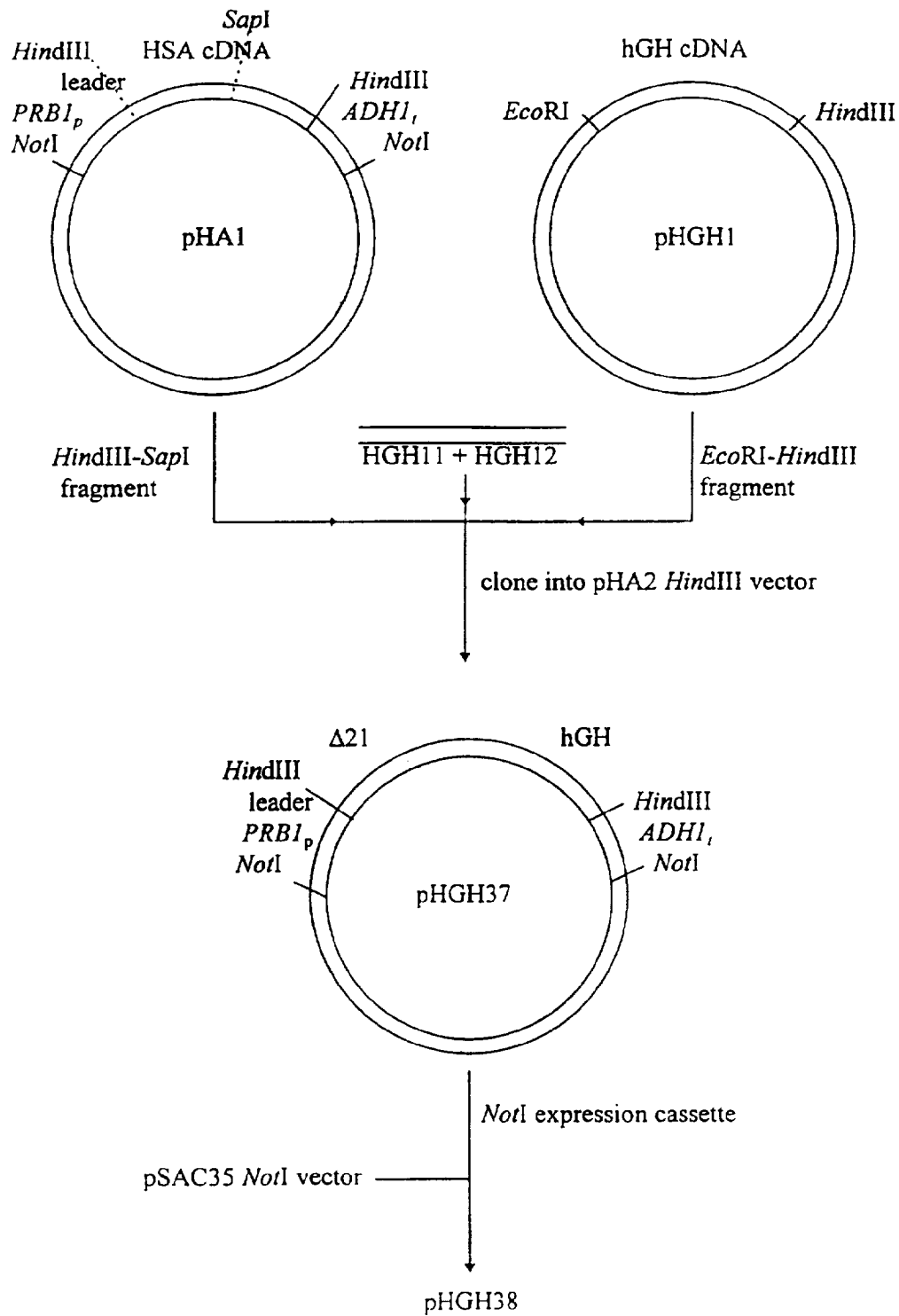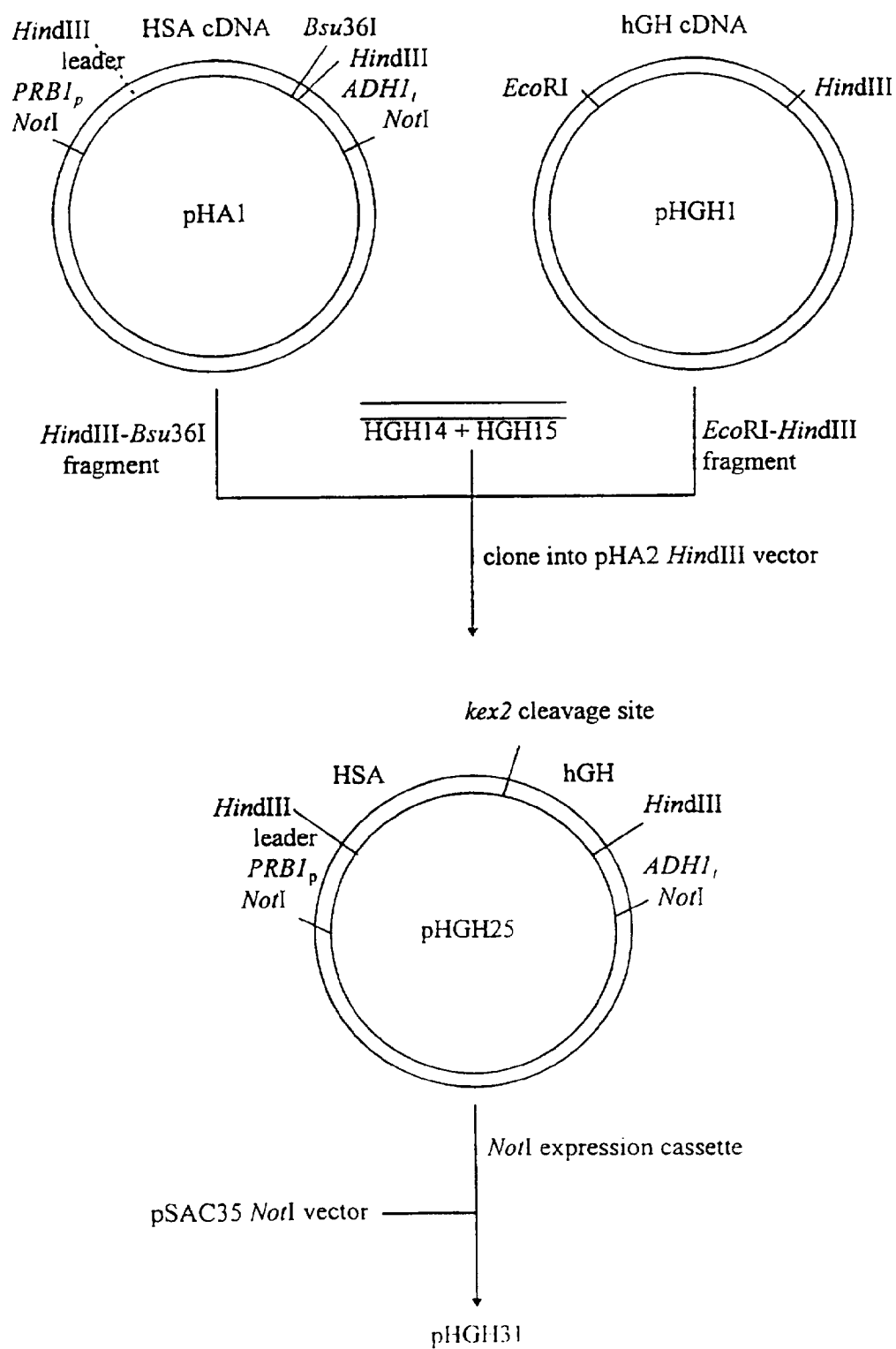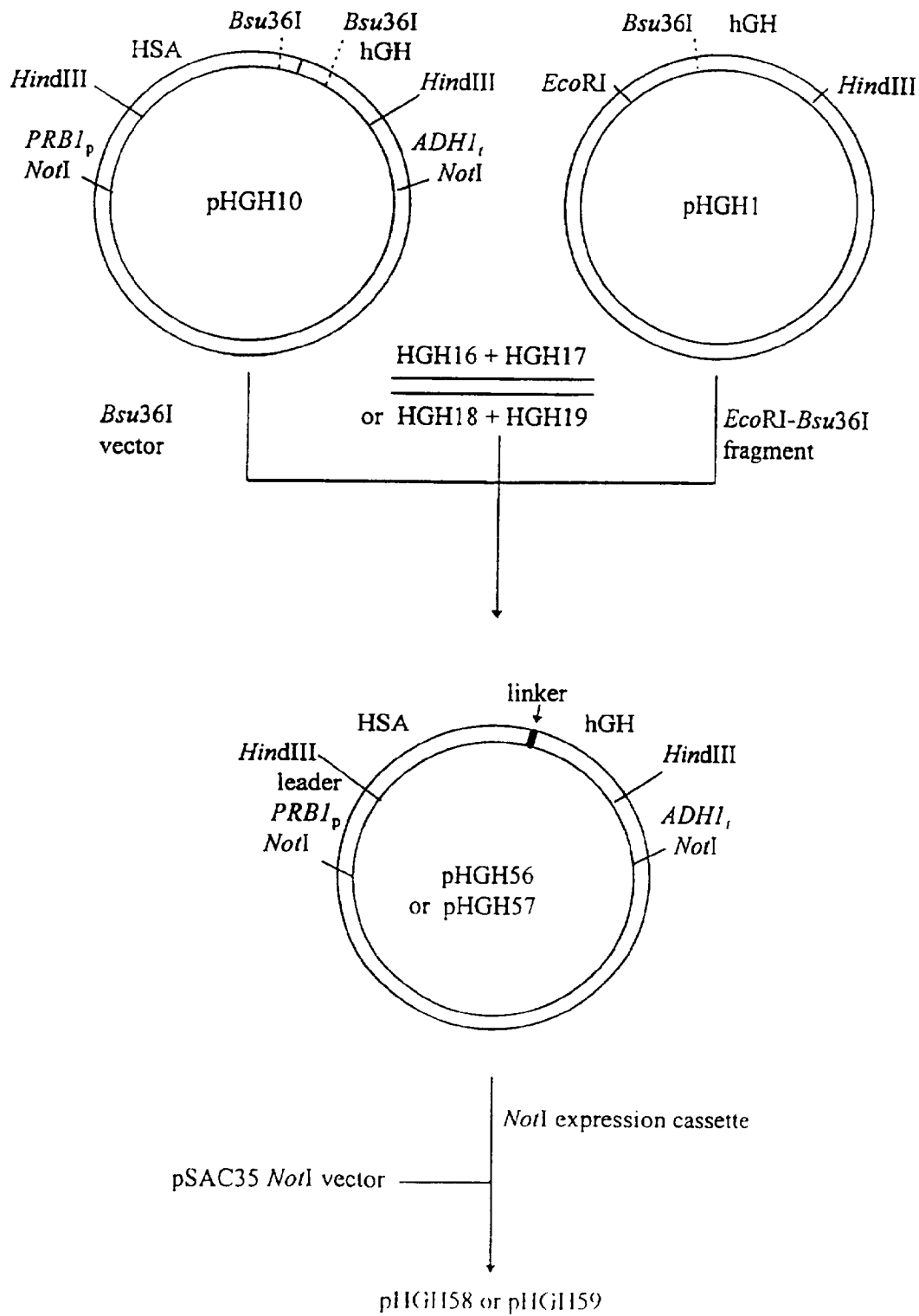
Figure 6 (cont'd)

Figure 7

Figure 8
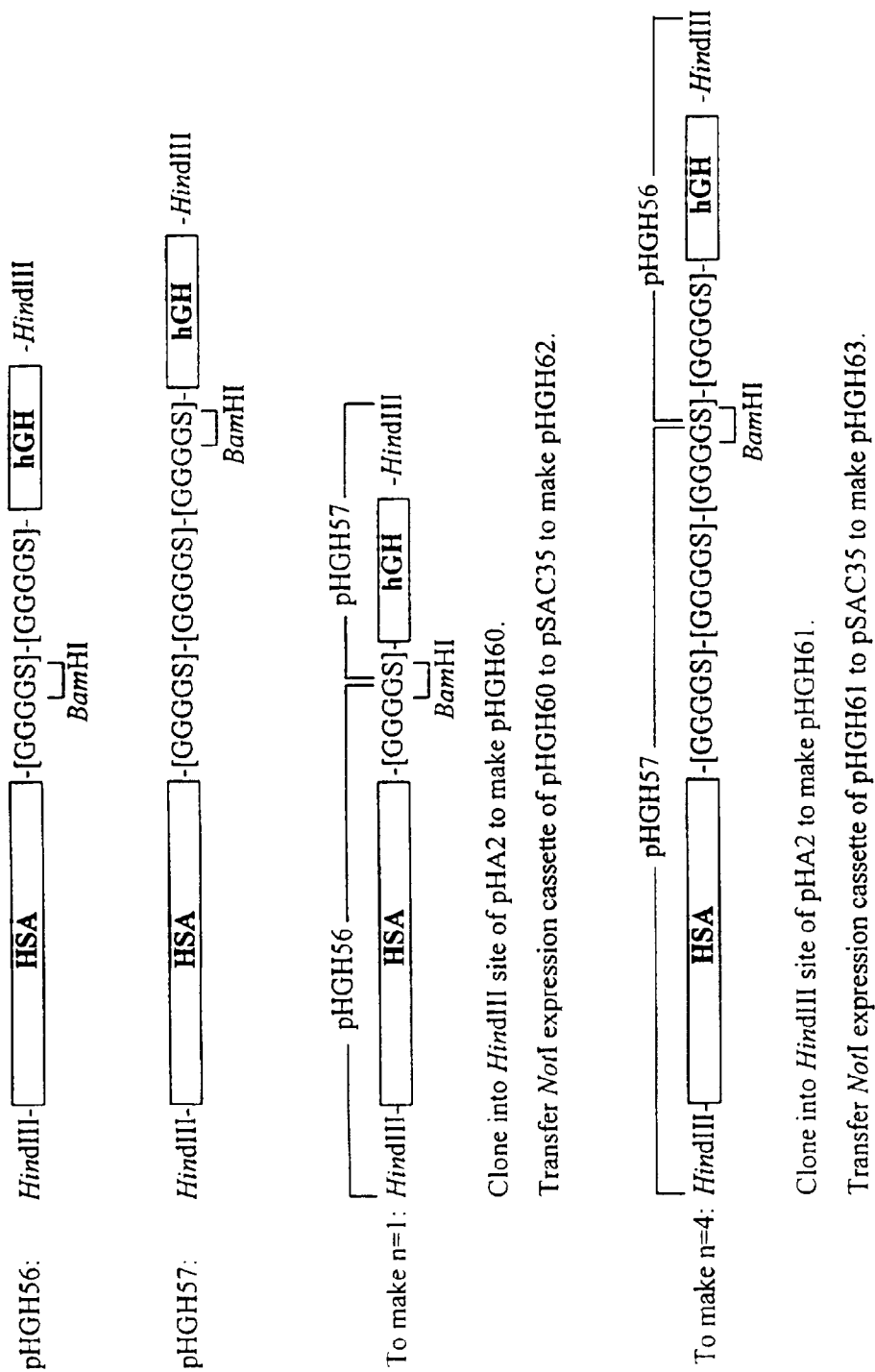
Figure 9

Figure 10

Figure 11

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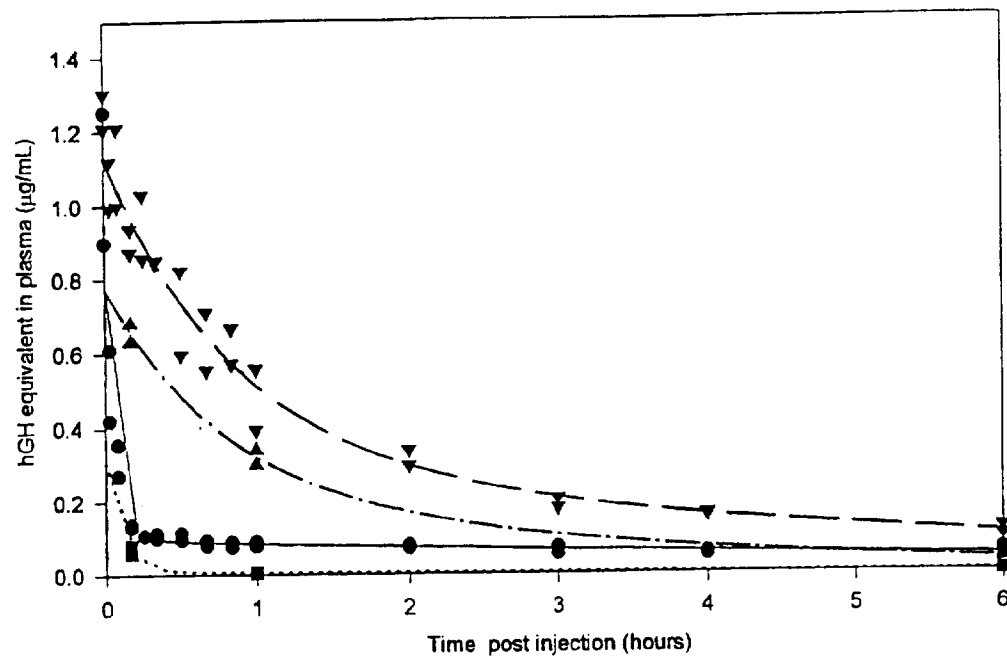
Figure 12

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/62   C12N15/14   C12N15/18   C07K14/765   C07K14/61
          A61K38/27   A61K38/38   C07K19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FEBS LETT, vol. 239, no. 1, October 1988, pages 18-22, XP000645058 M.J. POZNANSKY ET AL.: "Growth hormone-albumin conjugates." cited in the application see the whole document ---	1-21
Y	US 5 045 312 A (ASTON ROGER ET AL) 3 September 1991 see the whole document ---	1-21
Y	EP 0 319 641 A (GREEN CROSS CORP) 14 June 1989 see the whole document --- -/--	1-21

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 June 1997	1 3. 06. 97

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Hix, R |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 765 980 A (DEPRINCE RANDOLPH B ET AL) 23 August 1988 see the whole document ---	1-21
Y	WO 95 30759 A (RHONE POULENC RORER SA ;BECQUART JEROME (FR); CONSEILLER EMMANUEL) 16 November 1995 see the whole document ---	1-21
Y	WO 93 15199 A (RHONE POULENC RORER SA) 5 August 1993 see the whole document ---	1-21
Y	EP 0 399 666 A (DELTA BIOTECHNOLOGY LTD) 28 November 1990 cited in the application see the whole document ---	1-21
Y	APP. ENVIRON. MICROBIOL., vol. 56, no. 7, July 1990, pages 2125-2132, XP000618730 R. HIRAMATSU ET AL.: "The prepro-peptide of Mucor rennin directs the secretion of human growth hormone by Saccharomyces cerevisiae." cited in the application see the whole document ---	1-21
Y	BIOCHEMISTRY , vol. 59, no. 11, 1994, {MOSCOW}, pages 1247-1256, XP000618681 A.B. TSIOMENKO ET AL.: "Prosegment of yeast alpha-factor directs a heterologous protein {Human Growth Hormone} to the culture medium of Saccharomyces cerevisiae." cited in the application see the whole document ---	1-21
Y	GENES AND DEVELOPMENT, vol. 1, no. 3, May 1987, pages 268-76, XP000618715 C.A. PINKERT ET AL.: "An albumin enhancer located 10kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. " see the whole document -----	1-21

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INTERNATIONAL SEARCH REPORT

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:   19,20
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 19 and 20 (as far as it concerns a diseased state) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
     [ ] The additional search fees were accompanied by the applicant's protest.

     [ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5045312 A	03-09-91	NONE	
EP 0319641 A	14-06-89	CA 1326217 A DE 3884356 D DE 3884356 T ES 2059428 T US 5503993 A JP 2167095 A	18-01-94 28-10-93 10-03-94 16-11-94 02-04-96 27-06-90
US 4765980 A	23-08-88	NONE	
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WO 9315199 A	05-08-93	FR 2686899 A EP 0624195 A FI 943563 A JP 7503368 T	06-08-93 17-11-94 29-07-94 13-04-95
EP 0399666 A	28-11-90	DE 69002395 T EP 0407008 A ES 2060033 T WO 9013306 A	25-11-93 09-01-91 16-11-94 15-11-90